EXPERIMENTAL SUBCUTANEOUS COCCIDIOIDAL INFECTION IN THE MOUSE*

DEMOSTHENES PAPPAGIANIS, PH.D., CHARLES E. SMITH, M.D., ROBERT J. BERMAN, A.B. AND GEORGE S. KOBAYASHI, B.S.

Dermal manifestations of coccidioidomycosis occur in one of two forms: as the prognostically encouraging allergic erythema nodosum, or as the indicators of disseminated disease, verrucous skin lesions or subcutaneous abscesses which, if multiple, are prognostically unfavorable (1, 2). There were early impressions that these latter cutaneous lesions in which *Coccidioides immitis* can be demonstrated constituted primary coccidioidal infection (3, 4, 5); but it is recognized today that these lesions are secondary manifestations of systemic infection. Only two well documented cases of primary percutaneous coccidioidal infection have been reported (6, 7). In both, the site of inoculation was a finger and both incurred lymphangitis and lymphadenopathy of the arm. Wilson, *et al.* (6) isolated *Coccidioides immitis* not only from the primary ulcerated lesion, but also from the purulent fluid of the homolateral axillary nodes. There appeared to be no extension of the infection beyond the arm, and both patients recovered fully. Curtis (8) presented a preliminary report on what appeared to be a primary coccidioidal infection of the thigh.

Several early efforts were made to infect with *C. immitis* by percutaneous route. Rixford and Gilchrist (9) failed in an attempt to infect the unaffected skin of one of their patients with disseminated coccidioidomycosis. Rixford (3) produced dermal lesions in the dog by placing macerated tissue with spherules from a human lesion in contact with scarified skin. He also produced "chronic sores" which contained spherules, by inoculation into the skin of rabbits (4). Ophuls (10) reported that results of subcutaneous inoculations were "not at all constant." He produced a chronic ulcer at the site of inoculation on the leg of a dog, which led to abscess formation in the inguinal lymph nodes. Both lesions contained spherules and both sites healed completely after excision of the lesions. In a rabbit, subcutaneous inoculation gave a chronic ulcer which persisted for 6 months, but showed some tendency to heal. After excision of the lesion, the wound healed rapidly. There were no signs of generalized infection in the rabbit, nor in a similarly inoculated guinea pig sacrificed after 10 weeks. Similar results in the guinea pig were reported by Davis (11), and others (12, 13). Kawatsure (14) apparently found dissemination from the subcutaneous inoculation site in guinea pigs. More recently, Redaelli *et al.* (15) infected rats by subcutaneous inoculation with *C. immitis*, and found no lesions in the viscera except in rats which were treated with cortisone for 3 days before and 14 days after inoculation.

Wright *et al.* (16) studied the morphology of *C. immitis* in the subcutaneous "pneumoderma" and "granuloma pouch" of Selye (17) induced in rats. *C. immitis* could be isolated from the viscera between the fifth and twenty-third day. Lesions were observed on viscera of rats with the "granuloma pouch" from the 10th day onward, but none of the animals from either group appeared ill when sacrificed for autopsy.

The present work was an outgrowth of an attempt to determine whether the difference in virulence shown by two strains of *C. immitis* in the mouse would be associated with a differing histopathologic response.

MATERIALS AND METHODS

Two strains of *C. immitis*, strain Silveira, of high virulence by the intraperitoneal route, and strain 46, of very low virulence by the intraperitoneal route (18), were used in the first experiment. In the later experiments, only strain Silveira was employed. Suspensions of arthrospores were prepared by dispersing spores harvested from glucose-yeast extract agar in sterile 0.9 per cent saline.

* From the Naval Biological Laboratory, School of Public Health, University of California, Berkeley, California.

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The concentration of spores was adjusted to the desired value after pour plate viable counts had been carried out on Roessler's "natural" medium (19).

Male white mice (Namru strain) weighing 20 to 25 grams were inoculated subcutaneously (S.C.) with 0.1 ml of arthrospore suspension (approximately 100 viable spores) in the area of the right paralumbar fossa. The site of entry of the needle was marked with picric acid solution to facilitate subsequent identification of the site of the injection. At various times between 1 and 13 days, at 40 days, and at 90 days, mice were sacrificed. An area of the skin and subcutaneous tissue approximately 2 cm in diameter was excised, care being taken not to disrupt the membranes subjacent to the corium. The specimen thus removed was fixed in Technicon fixative FU-48®, and processed by the paraffin technic. Sections were stained either with hematoxylin and eosin or with periodic acid-Schiff's base (20).

In a second experiment, 15 mice were infected with 450 arthrospores of strain Silveira per animal. After 90 days, the animals were sacrificed and examined for visible lesions on the viscera and at the local subcutaneous site. Portions of the liver, spleen and lungs were inoculated onto 3 per cent malt agar (Difco). This malt agar was also used for culturing in the subsequent experiments.

A third experiment was carried out to study the comparative effect of intraperitoneal (I.P.) and subcutaneous (S.C.) inoculation of *C. immitis*. This involved two groups of 25 mice each of which was given 115 arthrospores of strain Silveira.

In a fourth experiment to study the immunologic response to I.P. challenge after S.C. primary infection, 25 mice were inoculated S.C. with strain Silveira as above. At the end of 70 days, 15 of the infected mice and 10 untreated control mice were challenged I.P. with 100 spores of *C. immitis*, strain Silveira. Five of the 15 previously inoculated mice were sacrificed 19 days after challenge along with the 10 mice which had been previously inoculated S.C., but not challenged I.P. These animals were examined for gross lesions and cultures made from the viscera and subcutaneous site of inoculation. Portions of the subcutaneous sites were removed for histologic examination as before. The remaining 10 animals which had initially been inoculated S.C. and which survived I.P. challenge were sacrificed after 30 days, examined and cultured as already indicated.

In a final experiment, 25 mice were given 2.5 mg of cortisone acetate (Cortone, Merck) in 0.1 ml. injected S.C. into the left paralumbar fossa on 3 successive days. The injections of this emulsion were made S.C. in the left dorsal area. Immediately following the third injection, the mice were inoculated S.C. on the right side with 100 spores of strain Silveira. A second group of 25 mice were simultaneously injected S.C. with 100 spores of strain Silveira without antecedent cortisone. Three mice were sacrificed from each group 1, 6, 9 and 15 days following subcutaneous inoculation. The mice were examined for evidence of visceral involvement; and cultures of lungs, spleens and livers were made to detect viable *C. immitis*.

RESULTS

In the initial experiment, after 5 to 10 days, both strains Silveira and 46 produced a local subcutaneous lesion demonstrable visibly and by palpation at the site of inoculation. Dissection of the areas revealed progressive degrees of reaction on the underside of the skin, depending on the time at which the mice were sacrificed. In general, the local site in those mice infected with strain 46 showed much less reaction. After 24 hours, it appeared slightly hyperemic. After 3 days, the hyperemic site had become swollen to a 1 mm nodule which adhered to the underside of the skin, but not to the underlying dorsal musculature. By 5 to 8 days, the lesions had increased to 2–4 mm hard nodules, which exuded purulent fluid when cut. The size of the local lesion increased by 1 or 2 mm during the ensuing 2 to 3 weeks. After 40 days, some of the abscesses appeared to contain less pus than earlier lesions. This was not a constant feature, for even after 90 days, the centers of some of the local abscesses yielded a small amount of purulent fluid. However, in some animals sacrificed at 90 days the lesion had decreased to a hard nodule less than 2 mm in diameter, while in others the local site was only slightly hyperemic or appeared normal.

Only rarely did the local lesion in mice infected with either strain Silveira or strain 46 ulcerate and form a scab. There was no other external evidence of illness in any animal throughout the 90 day period. There was, furthermore, no gross evidence of infection of the viscera. The localization of subcutaneously inoculated *C. immitis* is reflected in figure 1, which shows that intraperitoneal inoculation caused death of 88 per cent of the mice in 25 days, but that none of the subcutaneously inoculated group died during the 90 day observation period.

When the infecting dose was increased to 450 spores, the external appearance of the local site was unchanged. Of the 15 mice which had been

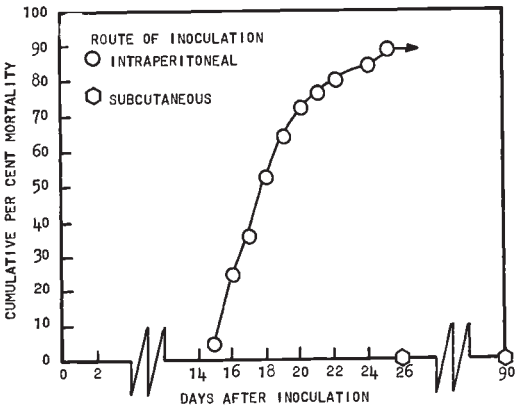

FIG. 1. Comparative virulence of *C. immitis* strain silveira following intraperitoneal and subcutaneous inoculation.

inoculated, 1 became ill and emaciated and died on the 17th day. Autopsy of this animal revealed widespread nodules characteristic of coccidioidal lesions throughout the lungs and in the liver and spleen. There was also a large soft white mass in the dorsal musculature underlying the intended site of injection. There was no nodule adherent to the under surface of the skin, and therefore it is very probable that the injection had penetrated the subcutaneous region and entered the dorsal muscles. The local lesion thus produced gave rise to dissemination of the fungus, followed by death in this animal. The 14 survivors, however, appeared healthy when sacrificed and autopsied at 90 days; however, 2 mice of this group contained scattered small (1 mm) nodules in the liver and a few in the lungs and spleen. The local lesion when present appeared to be slightly larger than those observed in mice inoculated with 100 spores. Six of the 14 animals had only slight hyperemia or no visible lesion at the local site of inoculation when sacrificed. The results of culturing the local site and the spleen, liver and lungs is shown in Table 1. Seven of the 14 mice contained viable *C. immitis* at the local subcutaneous site. Only 1 of the 2 mice with visible visceral lesions yielded viable *C. immitis* from these internal organs. Another mouse which did not have visible lesions yielded *C. immitis* from liver and lungs.

The pretreatment of mice with cortisone acetate did not reduce resistance to the subcutaneous inoculation of strain Silveira. Twelve of 25 mice which received 7.5 mg of cortisone acetate plus 100 spores subcutaneously remained active and healthy in appearance as did the untreated controls until sacrificed at 1, 6, 9 and 15 days. The 13 cortisone treated mice, which were not sacrificed until 90 days after inoculation, were similar in appearance to the controls. *C. immitis* was grown from the viscera of 3 of the cortisone-treated mice and 2 without cortisone and, in all other respects, culturing the local sites and organs indicated that cortisone did not materially increase dissemination from the local site.

Histologic observations. Sections taken from the subcutaneous inoculation sites generally showed the following: During the first week after inoculation, an active collection of polymorphonuclear neutrophils was the most conspicuous feature. Cells of *C. immitis* were infrequently observed and did not show maturation of spherules though some were 20 to 30 microns in diameter. Eight days after inoculation, there was loose aggregation of polymorphonuclear leukocytes within the subcutaneous fat layer and above the areolar membranes. Polymorphonuclear "band" cells predominated, and could be seen collected in intensely stained zones within the larger aggregation. This early abscess with some necrosis already in evidence also showed a mononuclear response and young fibroblasts were evident at

TABLE 1

Recovery of viable C. immitis *from mice following subcutaneous inoculation of 450 arthrospores*

Mouse	Tissue Cultured†			
	Sub-cutaneous	Spleen	Liver	Lungs
1	−	−	−	−
2	−	−	+	+
3	−	−	−	−
4	+	−	−	−
5	−	−	−	−
6	+	−	−	−
7	−	−	−	−
8	+*	*	*	*
9	+	−	−	−
10	+	−	−	−
11	−	−	−	−
12	+	−	−	−
13	+	+	+	+
14	−	−	−	−

* Bacterial growth in cultures; *C. immitis* did not grow in cultures of the organs.

† Mice sacrificed 90 days after inoculation.

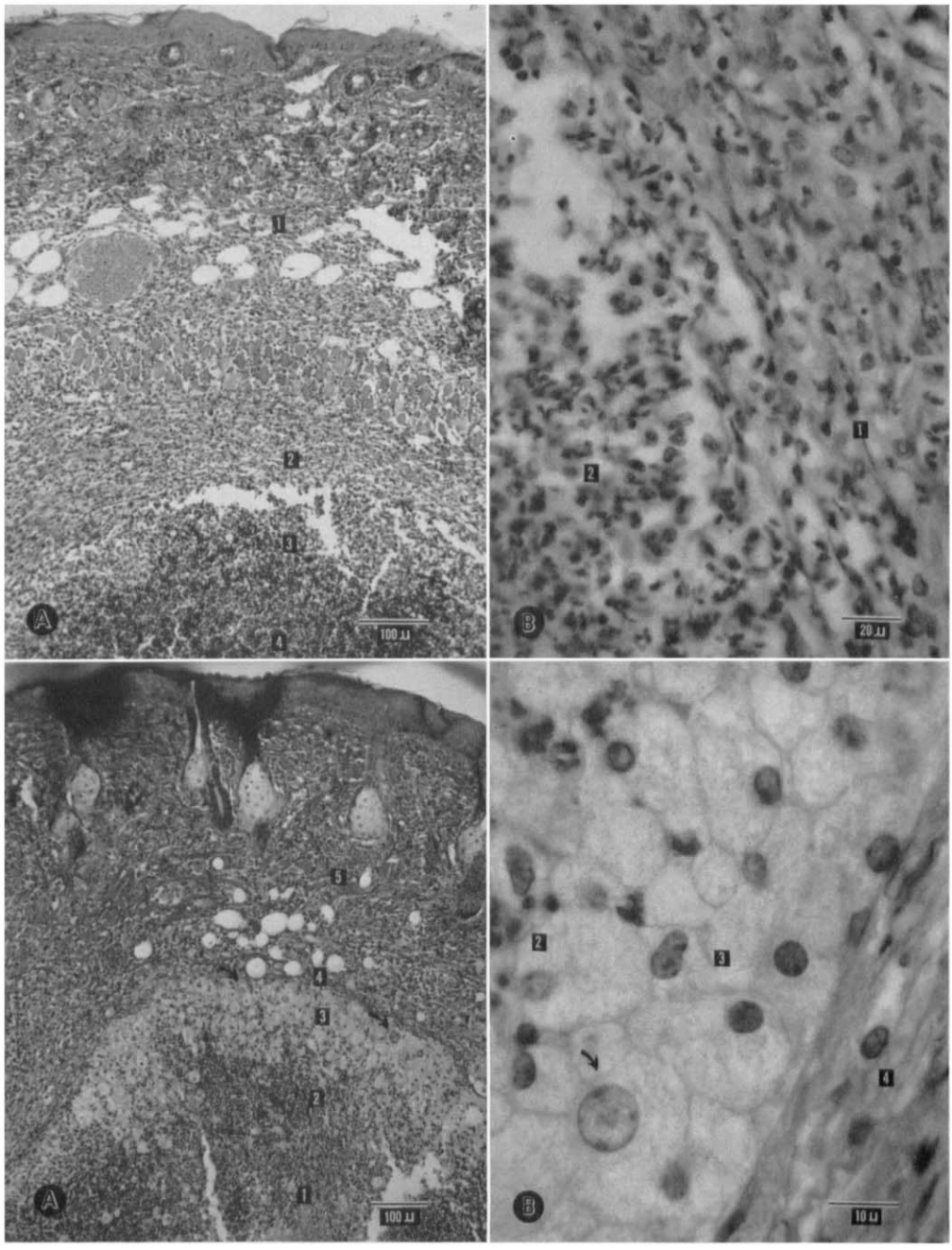

FIG. 2A. (Upper left) Subcutaneous coccidioidal lesion at 13 days. The following zones were apparent from microscopic examination: 1. Compressed (now extracted) adipose layer; 2. fibroblasts and strands of collagen plus lymphocytes and mononuclear cells; 3. and 4. internal area of polymorphonuclear leucocytes with necrosis. Hematoxylin and eosin.

FIG. 2B. (Upper right) Higher power view of margin of lesion shown in A. Zone 1 shows the already well formed fibrous capsule; and 2. the acute polymorphonuclear reaction of the early abscess. Hematoxylin and eosin.

FIG. 3A. (Lower left) Subcutaneous coccidioidal lesion at 40 days. 1. Central, soft necrotic area; 2. polymorphonuclear and macrophagic cells; 3. spongy epithelioid cells (arrows show numerous immature *C. immitis* spherules); 4. fibrous wall and 5. collagen fibers. Hematoxylin and eosin.

FIG. 3B. (Lower right) Higher power view of margin of lesion with portion of zone 2, polymorphonuclear and macrophagic cells; 3. epithelioid zone with one immature spherule (arrow) and 4. the well formed fibrous strands of the border. Hematoxylin and eosin.

the periphery. Young *Coccidioides* spherules were seen, some with peripherally collected cytoplasm, others with their cytoplasm condensed centrally, detached from the spherule wall.

By the 13th day, the abscessed area had compressed adipose cells between which could be seen the peripatetic inflammatory cells outside the abscess proper. The abscess was bordered by aggregated fibroblasts and strands of collagen intermingled with lymphocytes and macrophages. Next inward, were zones consisting mainly of polymorphonuclear leukocytes which gave the stained lesion its dark color, and centrally was a large mass of necrotic debris including nuclear fragments (Fig. 2). Note that only the very edge of this necrotic area is visible in figure 2A. The microscopic architecture described above was found in abscesses caused by Silveira which produced marked necrosis, and many fungus cells; strain 46, however, produced smaller abscesses with less necrosis and only occasionally were *Coccidioides* cells found. Mature spherules with endospores were very rare and found only in lesions produced by strain Silveira.

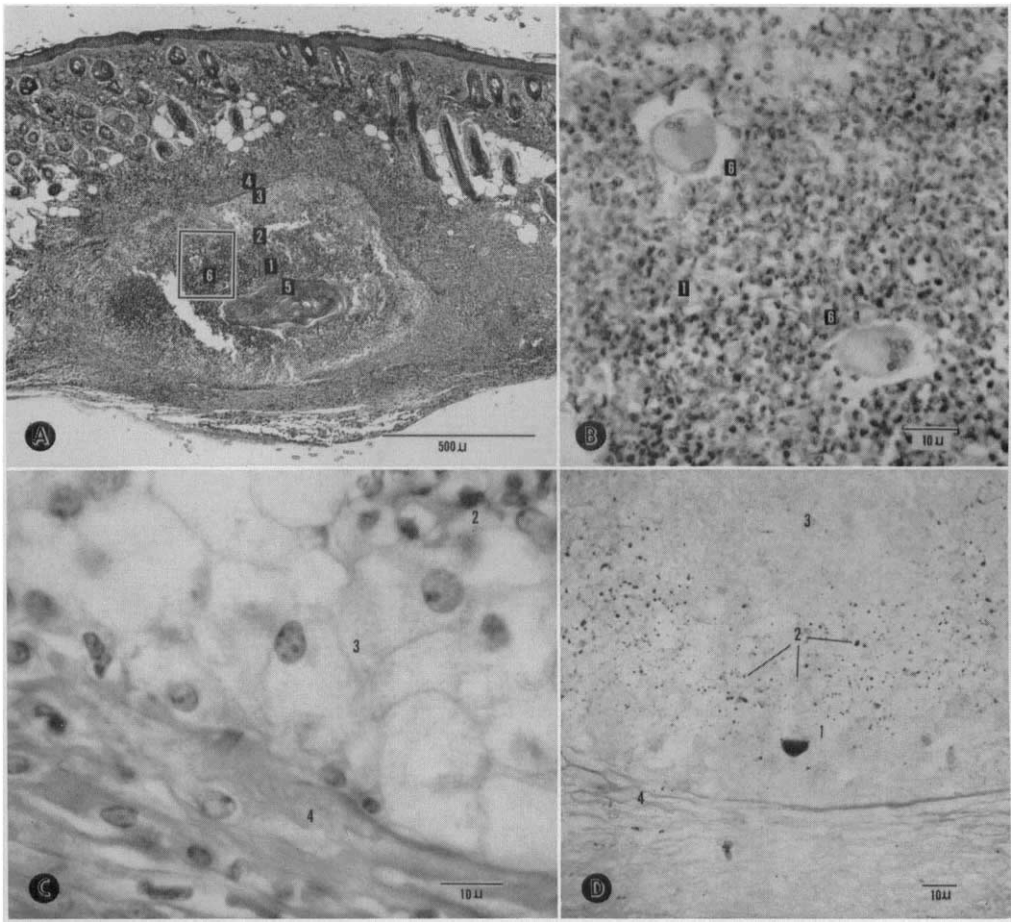

FIG. 4A. Subcutaneous coccidioidal lesion at 90 days. 1. Necrotic area developing mainly from polymorphonuclear response; 2. polymorphonuclear and mononuclear cells; 3. spongy epithelioid zone; 4. fibrous abscess wall; 5. eosinophilic ("fibrinoid") aggregate; 6. two giant cells. Hematoxylin and eosin.

FIG. 4B. The two giant cells 6 are shown in higher magnification in the turbulent area 1 of polymorphonuclear cell collection and necrosis. Hematoxylin and eosin.

FIG. 4C. An area at the margin of the lesion of A showing 2. a part of the central polymorphonuclear and mononuclear zone; 3. epithelioid cells; and 4. fibrous capsule. Hematoxylin and eosin.

FIG. 4D. 1. Collapsed spherule in belt 2 of Schiff positive fragments of spherules or endospores at edge of degenerative central mass 3 of inflammatory cells (not stained); 4. connective tissue fibers in abscess wall. Periodic acid-Schiff stain.

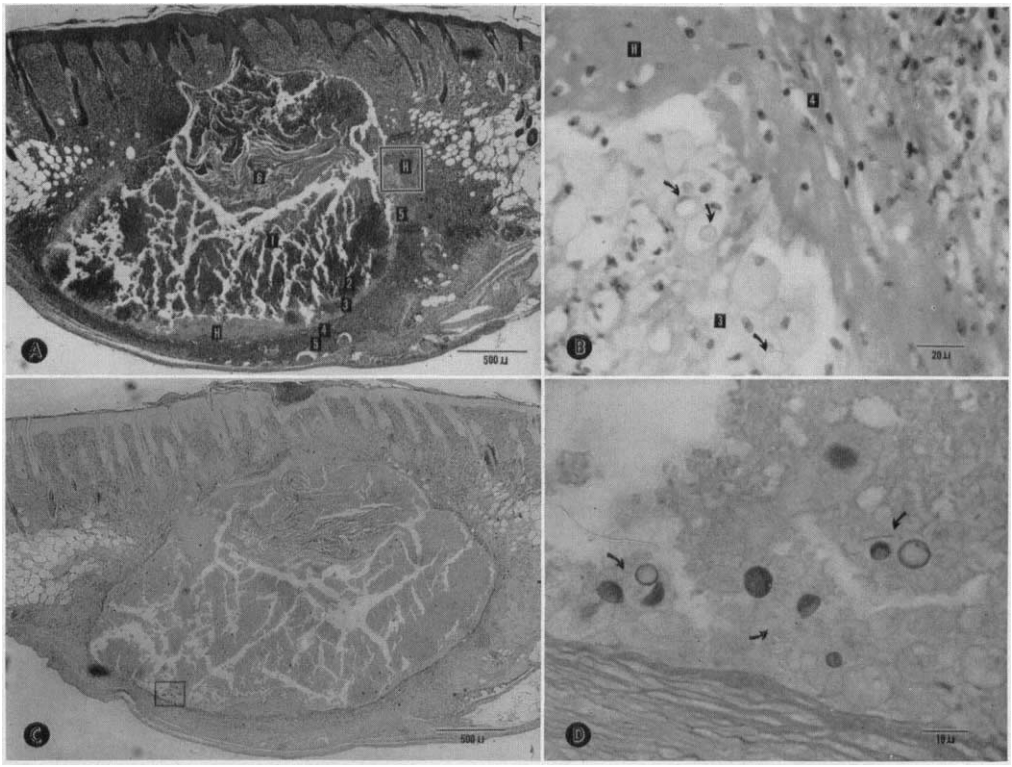

FIG. 5A. Subcutaneous coccidioidal lesion at 90 days. Pronounced area 1 of necrosis in center of abscess; 2. dark zone of polymorphonuclear cells; 3. vesiculated epithelioid cells; 4. and 5. fibrous capsule and collagen fibers in wall of abscess; 6. eosinophilic ("fibrinoid") aggregate. H is hyaline eosinophilic zone. Hematoxylin and eosin.

FIG. 5B. Higher magnification of area shown in A. At 3 is the zone of vesiculated epithelioid cells; H is hyaline area within fibrous capsule 4. (Arrows indicate immature spherules). Hematoxylin and eosin.

FIG. 5C. Section adjacent to that in A stained with periodic acid-Schiff stain. Dark bodies are collapsed or degenerate spherules.

FIG. 5D. Enlargement of zone outlined in C, showing several collapsed spherules or spherule walls inside the fibrous shell of the abscess. Periodic acid-Schiff stain.

At 40 days the reaction caused by strain 46 included much less necrosis than was induced by strain Silveira. The lesion induced by strain 46 consisted of connective tissue elements, mononuclear cells and capillaries (granulation tissue) which extended to the under side of the epidermal layer. There was little necrosis but packed epithelioid cells were found throughout the lesion. Only empty shells or cell walls of strain 46 could be found. Strain Silveira, however, induced relatively large (up to 3 mm) abscessed areas. These contained a necrotic center of purulent fluid perhaps with caseous material admixed, surrounded by masses of both polymorphonuclear and mononuclear cells. Encircling the latter was a striking rim of large vesiculated or finely granular cells (Fig. 3). These appeared to be epithelioid cells, but the possibility that they were degenerate fat cells was also considered*. This layer of large cells lay adjacent to and inside the fibrous wall containing conspicuous collagen fibers which surrounded the entire lesion. There were also broad zones of eosinophilic hyaline material (scar tissue) with nuclei scattered throughout. These were found on the inner border of the fibrous capsule. Many immature and rather unhealthy appearing spherules were seen with their cytoplasm condensed centripetally.

When sections were examined from 90 day lesions (Fig. 4 and 5), there was little qualitative difference from what was noted at 40 days. Some

* Attempts to identify these large spongy cells by the use of frozen sections and Sudan IV gave inconclusive results, but indicated that they were not fat cells. However, fatty material interspersed within the fibrous capsule of the abscesses formed a complete circle around the lesion.

aspects were accentuated, for example the central eosinophilic strands, and granulation tissue external to the fibrous capsule. The honeycomb layer of foamy epithelioid cells adjacent to the fibrous capsule was very conspicuous and occasionally some of these cells appeared to have coalesced into small multinucleate giant cells (Fig. 4A and B). Spherules when visible, were immature degenerate types. In some instances they could be detected only with the periodic acid-Schiff stain. Then they appeared as single widely scattered, collapsed or crenated bodies (Fig. 5C and D). As shown in figure 4D, the periodic acid-Schiff stain also revealed in some lesions only a rare intact but immature spherule with a great number of fragments taking the Schiff stain. Some of these may be freed endospores; however, some are extremely small polysaccharide remnants. These are suggestive of a coarser counterpart of Huntington's (21) "Schiff-positive dust" in human coccidioidal lesions.

Only 3 or 4 endospore-containing spherules were found in all the sections examined from subcutaneous lesions. However, the numbers of organisms observed indicated that possibly some maturation with endospore formation and liberation occurred early after inoculation of the mice. The sparse maturation of spherules reflected the active coccidioidostatic environment of the subcutaneous region in the normal mouse.

By contrast, the death of the single mouse previously described following the presumed subcutaneous inoculation of 450 spores was preceded by massive destruction of dorsal musculature into which the arthrospores were probably deposited (Fig. 6A). The lungs of this animal showed the complete disorganization of pulmonary architecture and the prolific mature spherule formation usually associated with coccidioidal disease in the mouse (Fig. 6B). The fibrous wall so evident at the periphery of the subcutaneous lesions was absent in this animal. In general the microscopic

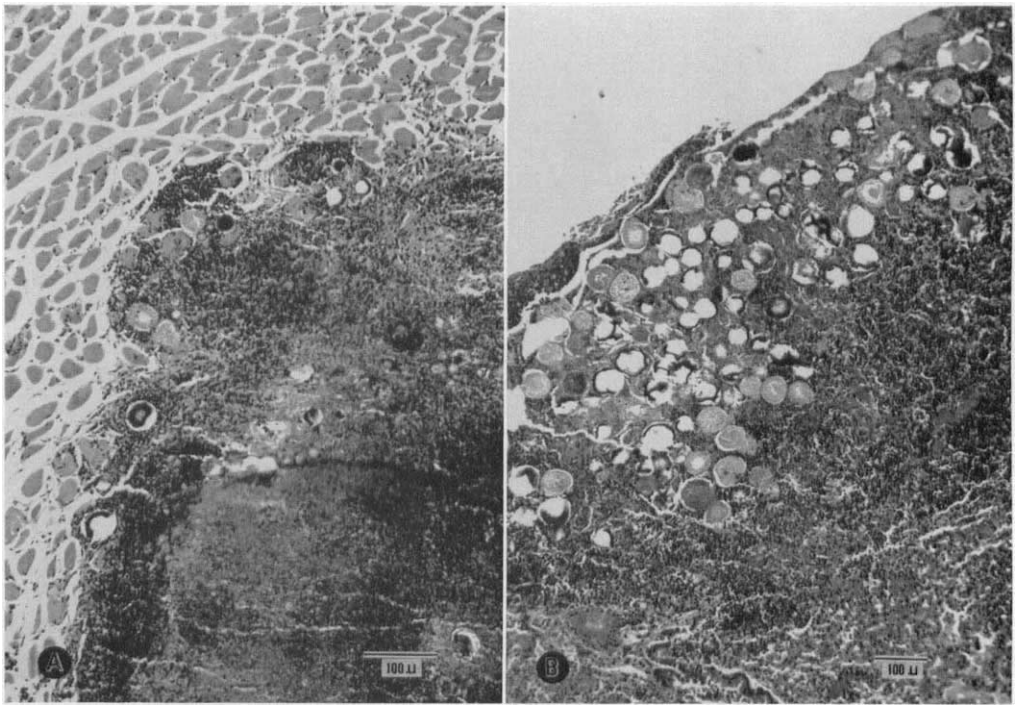

FIG. 6A. Coccidioidal lesion in mouse which died 17 days after presumed subcutaneous inoculation. Muscle of dorsum has undergone widespread necrosis and contains abundant inflammatory cells. Several mature endospore filled spherules are visible. Hematoxylin and eosin.

FIG. 6B. Diseased portion of lung of mouse which died 17 days after inoculation (see Fig. 6A). Extensive disruption of pulmonary tissue with necrosis and hemorrhage. Many mature spherules with endospores can be seen. Hematoxylin and eosin.

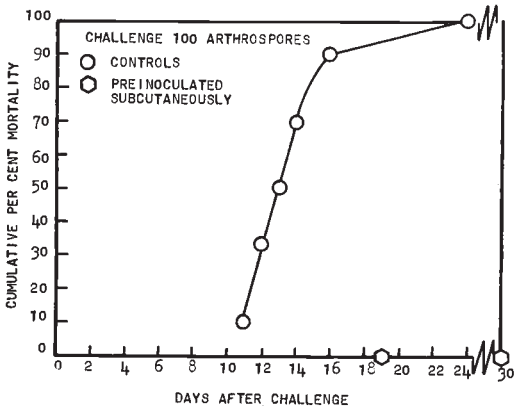

FIG. 7. Resistance of subcutaneously inoculated mice to subsequent intraperitoneal challenge with *C. immitis* strain silveira.

picture described above is similar to that observed in the rat by Redaelli *et al.* (15).

Immunogenic effect. Immunization of the mice by the subcutaneous inoculation of viable virulent spores of strain Silveira was shown by the results shown in figure 7. At autopsy of those mice which resisted intraperitoneal challenge, only 1 or 2 small discrete whitish tubercles were observed on the liver, spleen or diaphragm. These lesions were not over 1 to 2 mm in size but were found to contain viable organisms on culture. Variable survival of the fungus was noted in the subcutaneous lesions, and it is believed that the visceral lesions contained organisms derived from the intraperitoneal challenge dose. In other experiments, subcutaneously inoculated mice which were subsequently challenged with 100 spores by intraperitoneal route showed 100 per cent survival for periods in excess of 90 days.

Further studies to be reported elsewhere have demonstrated the efficacy of the subcutaneous injection of viable *C. immitis* in engendering an immune response to intranasal as well as intraperitoneal challenge with 100 spores (22).

DISCUSSION

In its marked capacity to localize *C. immitis* when this organism is injected subcutaneously, the mouse shows a remarkable difference from its susceptibility to the lethal effects when *C. immitis* is administered by other routes. Although in many instances viable fungus cells were present in the local subcutaneous abscesses, extension of the organism to the viscera was rare and not accompanied by lethal disease. Wright *et al.* (16) also found extension of *C. immitis* to the lungs, livers, spleens and kidneys of rats inoculated by way of subcutaneous granuloma pouches or pneumodermata, but their animals likewise appeared to remain healthy. Other examples of the localization of *C. immitis* in normal experimental animals were presented in the introduction, and it would appear that the skin of many different species including man has a significant coccidioidostatic effect. Even the administration of cortisone acetate for 2 days prior to and on the same day as the subcutaneous inoculation caused only infrequent dissemination from the primary lesion, while a similar regimen had a pronounced enhancing effect on intraperitoneal infection with a relatively avirulent strain (23). In the work of Redaelli *et al.* (15), cortisone administered to rats for 3 days prior to inoculation and 14 days after, brought about dissemination from the subcutaneous site to the viscera.

More extensive studies will be necessary to identify possible cellular characteristics of the subcutaneous sites which inhibit *C. immitis* and promote the organization of the wall of fibrous tissue so lacking in disseminated coccidioidal lesions in other organs of the mouse. On the other hand, the stormy polymorphonuclear response of the subcutaneous site is not unique and occurs in most coccidioidal lesions of the mouse regardless of organ. Adding to the "mixed granulomatous" (24) picture of the subcutaneous abscesses were the large vesiculated epithelioid cells lining the inner wall and extending into the central necrotic debris of the 40 and 90 day lesions. These cells would appear to have a secondary importance in the temporal sense, since their honeycomb arrangement was not conspicuous in the early lesions at 8 or 13 days, at which time an encircling wall of fibroblastic tissue had already become apparent. While staining with Sudan IV did not demonstrate an adipoid nature of the so-called "epithelioid" cells, the sections of the abscesses did show peripheral distribution of lipid. The possible significance of subcutaneous fat in arresting the development of *C. immitis* is unknown, but the presence of intraperitoneal depot fat does not appear to retard the fungus inoculated by that route.

The localized disease resulting from accidental percutaneous inoculation of humans with *C. immitis* as presented by Wilson *et al.* (6) and Trimble and Doucette (7) were not amenable to serial histopathologic study. The former authors

obtained tissue five weeks after the onset of the infection, at a time when the local lesion had undergone considerable regression. They found some areas predominantly composed of polymorphonuclear cells with eosinophiles and lymphocytes, while in others small lymphocytes and giant cells were conspicuous. *Coccidioides* spherules were sparse and difficult to find. Trimble and Doucette examined a biopsy specimen taken from the infected site on the 13th day of illness. In conformity with the earlier stage of this lesion, they found mainly an acute inflammatory reaction with polymorphonuclear leukocytes and lymphocytes, with rare epithelioid and giant cells, but numerous spherules; some with endospores could be seen. Thus, in both man and mouse, the occurrence of limited primary coccidioidal infections in the skin may have parallel features although a thorough histologic comparison cannot yet be made.

Increased resistance to challenge with *C. immitis* following subcutaneous inoculation of viable cells is in conformity with the immune status of humans with a history of previous exposure (2, 24). These preliminary findings (22) suggesting that viable cells are more efficacious than formalin killed cells in inducing resistance to challenge with *C. immitis*, invite further attention to experimental subcutaneous inoculation of *C. immitis*.

SUMMARY

Subcutaneous inoculation of 100 arthrospores of two strains of *Coccidioides immitis* into mice resulted in localized abscesses. Intraperitoneal inoculation of a similar dose of the virulent strain resulted in progressive lethal disease. The abscesses resulting from subcutaneous injection of the virulent strain showed more extensive necrosis than was observed with the relatively avirulent strain. A fibroblastic wall at the periphery of the lesion had formed by 8 and 13 days. This wall appeared to have an enclosing effect on the intense early polymorphonuclear response and necrosis which followed. At 40 and 90 days similar lesions were observed consisting of a necrotic purulent or caseous center, a zone of polymorphonuclear and mononuclear cells, a rim of spongy epithelioid cells, all surrounded by a fibrous capsule and "granulation" tissue. Viable *C. immitis* could be cultured from some of these local abscesses, but not from others, and the periodic acid-Schiff stain revealed many degenerate fungus cells or fragments thereof. Cortisone acetate just prior to and at the time of challenge did not increase dissemination from the local lesion to the viscera. Mice which produced the local abscess after subcutaneous inoculation with the virulent strain were subsequently challenged with virulent *C. immitis* by the intraperitoneal route and were found to be resistant. Preliminary results also indicated an increased resistance to intranasal challenge.

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ANNOUNCEMENT

SCIENTIFIC EXHIBITS

Applications are now being received for space for scientific exhibits at the meeting of the American Academy of Dermatology at the Palmer House, Chicago, in December, 1959.

Awards will be given for the best exhibits in each of the following categories:

(1) Original investigation
(2) Teaching value
(3) Historical

Please address all correspondence before the application deadline, June 1, 1959, to Frederick D. Malkinson, M.D., Section of Dermatology, University of Chicago Clinics, 950 E. 59th St., Chicago 37.